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(71) Applicant *(for all designated States except US)*: ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: O'CONNELL, Ann, Patricia, Josephine [IE/GB]; Jealott's Hill Research Station, Bracknell, Berkshire RG42 6ET (GB).

(74) Agents: HUSKISSON, Frank, Mackie et al.; Zeneca Agrochemicals, Intellectual Property Dept., Jealott's Hill Research Station, P.O. Box 3538, Bracknell, Berkshire RG42 6YA (GB).

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(54) Title: METHOD FOR MODULATING THE BIOMASS OF PLANTS

(57) Abstract

This invention relates to a method of modulating the biomass of plants comprising incorporating into the genome of a said plant a DNA which modulates the production or function of an endogenous plant cinnamoyl CoA reductase gene (CCR). The gene for CCR is involved in the production of lignin in plants. Its modulation can be obtained by gene silencing via sense or antisense downregulation or cosuppression.

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METHOD FOR MODULATING THE BIOMASS OF PLANTS

The present invention relates to a method of modulating the biomass of plants.

Biomass is defined as the direct or indirect accumulation of biological material. This can have effects on starch, oils, cellulose and other material and it encompasses the total accumulation of matter. In plant species cellulose accounts for a high percentage of the total biomass and it is intimately linked to lignins. The cinnamoyl CoA reductase enzyme (CCR) in plants catalyses the first reductive step of the lignin biosynthetic pathway, the conversion of the hydroxycinnamoyl CoA esters (*p*-coumaroyl-Co A, feruloyl-CoA and sinapoyl-CoA) to their corresponding aldehydes. It is the first enzyme dedicated to the synthesis of lignin precursors. Lignin is a complex aromatic bipolymer which waterproofs, reinforces and maintains structural integrity of plant secondary cell walls (Boudet et al., 1995). Lignin has a role in the structure and development of plants and represents a major component of the terrestrial biomass and is deemed to have great economic and ecological significance (Brown, 1985, Journal of Applied Biochem. 7. pp. 371-387). When exploiting the biomass of certain fodder crops, lignin is a limiting factor with regard to the digestibility and nutritional yield. It has been clearly demonstrated that the digestibility of fodder crops by ruminants, is inversely proportional to the lignin levels of the plants (Cherney et al., 1991).

Two principal methods for suppressing the expression of endogenous genes are known. These are referred to in the art as "antisense downregulation" and "sense downregulation" or "cosuppression". Both of these methods can lead to an inhibition of expression of the target gene, often referred to as "gene-silencing". In addition to this overexpression may be achieved by insertion of one or more extra copies of the selected gene. Other lesser used methods involve modification of the genetic control elements, the promoter and control sequences, to achieve greater or lesser expression of an inserted gene. In antisense downregulation, a DNA which is complementary to all or part of the target gene is inserted into the genome in reverse orientation and without its translation initiation signal. The simplest theory is that such an antisense gene, which is transcribable but not translatable, produces messenger RNA (mRNA) which is complementary in sequence to the mRNA product transcribed from the endogenous gene. That antisense mRNA then binds with the naturally produced "sense" mRNA to form a duplex which inhibits translation of the natural

mRNA to protein. It is not necessary that the inserted antisense gene be equal in length to the endogenous gene sequence, a fragment is sufficient. The size of the fragment does not appear to be particularly important. Fragments as small as 40 or so nucleotides have been reported to be effective. Generally somewhere in the region of 50 nucleotides is accepted as sufficient to obtain the inhibitory effect. However, it has to be said that fewer nucleotides may very well work: a greater number, up to the equivalent of full length, will certainly work. It is usual simply to use a fragment length for which there is a convenient restriction enzyme cleavage site somewhere downstream of fifty nucleotides. The fact that only a fragment of the gene is required means that not all of the gene need be sequenced. It also means that commonly a cDNA will suffice, obviating the need to isolate the full genomic sequence. There are however instances where the genomic sequences may be desired as the intron sequences may also be used for the construction of gene silencing vectors.

The antisense fragment does not have to be precisely the same as the endogenous complementary strand of the target gene. There simply has to be sufficient sequence similarity to achieve inhibition of the target gene. This is an important feature of antisense technology as it permits the use of a sequence which has been derived from one species to be effective in another and obviates the need to construct antisense vectors for each individual species of interest. Although sequences isolated from one species may be effective in another, it is not infrequent to find exceptions where the degree of sequence similarity between one species and the other is insufficient for the effect to be obtained. In such cases, it may be necessary to isolate the species-specific homologue.

Antisense downregulation technology is well-established in the art. It has been the subject of several textbooks and many hundreds of journal publications. The principal patent reference is European Patent No. 240,208 in the name of Calgene Inc. There is no reason to doubt the effectiveness of antisense technology. It is well-established, used routinely in laboratories around the world and products in which it is used are on the market.

Both overexpression and downregulation can be achieved by "sense" technology. If a full length copy of the target gene is inserted into the genome then a range of phenotypes is obtained, some "overexpressing" the target gene, some "underexpressing". A population of plants produced by this method may then be screened and individual phenotypes isolated.

As with antisense, the inserted sequence is lacking in a translation initiation signal. Another

similarity with antisense is that the inserted sequence need not be a full length copy. Indeed, it has been found that the distribution of over- and under- expressing phenotypes is skewed in favour of underexpression and this is advantageous when gene inhibition is the desired effect. For overexpression, it is necessary that the inserted copy gene retain its translation initiation codon. The principal patent reference on cosuppression is European Patent 465,572 in the name of DNA Plant Technology Inc. There is no reason to doubt the operability of sense/cosuppression technology. It is well- established, used routinely in laboratories around the world and products in which it is used are on the market.

Sense and antisense gene regulation is reviewed by Bird and Ray in Biotechnology and Genetic Engineering Reviews 9: 207-227 (1991). The use of these techniques to control selected genes in tomato has been described by Gray et.al., (1992) in *Plant Molecular Biology*, volume 19, pages 69-87.

Gene control by any of the methods described requires insertion of the sense or antisense sequence, with appropriate promoters and termination sequences containing polyadenylation signals, into the genome of the target plant species by transformation, followed by regeneration of the transformants into whole plants. It is probably fair to say that transformation methods exist for most plant species or can be obtained by adaptation of available methods.

For dicotyledonous plants the most widely used method is *Agrobacterium*- mediated transformation. This is the best known, most widely studied and, therefore, best understood of all transformation methods. The rhizobacterium *Agrobacterium tumefaciens*, or the related *Agrobacterium rhizogenes*, contain certain plasmids which, in nature, cause the formation of disease symptoms, crown gall or hairy root tumours, in plants which are infected by the bacterium. Part of the mechanism employed by *Agrobacterium* in pathogenesis is that a section of plasmid DNA which is bounded by right and left border regions is transferred stably into the genome of the infected plant. Therefore, if foreign DNA is inserted into the so-called "transfer" region (T-region) in substitution for the genes normally present therein, that foreign gene will be transferred into the plant genome. There are many hundreds of references in the journal literature, in textbooks and in patents and the methodology is well-established.

Various methods for the direct insertion of DNA into the nucleus of monocot cells are known.

In the ballistic method, microparticles of dense material, usually gold or tungsten, are fired at high velocity at the target cells where they penetrate the cells, opening an aperture in the cell wall through which DNA may enter. The DNA may be coated on to the microparticles or may be added to the culture medium.

In microinjection, the DNA is inserted by injection into individual cells via an ultrafine hollow needle.

Another method, applicable to both monocots and dicots, involves creating a suspension of the target cells in a liquid, adding microscopic needle-like material, such as silicon carbide or silicon nitride "whiskers", and agitating so that the cells and whiskers collide and DNA present in the liquid enters the cell.

In summary, then, the requirements for both sense and antisense technology are known and the methods by which the required sequences may be introduced are known. What remains, then is to identify genes whose regulation will be expected to have a desired effect, isolate them or isolate a fragment of sufficiently effective length, construct a chimeric gene in which the effective fragment is inserted between promoter and termination signals, and insert the construct into cells of the target plant species by transformation. Whole plants may then be regenerated from the transformed cells.

The purification and characterisation of cinnamoyl CoA: Oxidoreductase in *Eucalyptus gunnii* is detailed by Goffner *et al* in Plant Physiol. (1994), volume 106, pages 625-632. The use of DNA sequences to regulate the lignin levels of plants is the subject of the published patent application WO 9527790, in the name of Centre National de la Recherché Scientifique.

An object of the present invention is to provide a method for the production of transgenic plants having a biomass which is altered when compared with an untransformed control.

According to the present invention there is provided a method of modulating the biomass of a plant comprising incorporating into the genome of a said plant a DNA which modulates the production or function of an endogenous plant cinnamoyl CoA reductase gene.

Preferably the DNA has a substantially similar nucleotide sequence to an endogenous plant cinnamoyl CoA reductase gene. The said DNA may be inserted into the plant in sense or antisense orientation.

Alternatively or additionally the DNA is a nucleotide sequence which is substantially similar to an endogenous plant cinnamoyl CoA reductase enzyme inhibitor.

Also according to the present invention there is provided a gene construct comprising in sequence a promoter which is operable in a target plant, a coding region which is substantially similar to an endogenous plant cinnamoyl CoA reductase gene and a termination sequence which is operable in a target plant.

The promoter may be switchable or inducible or tissue, organ or fruit specific and particularly the promoter may be cauliflower mosaic virus.

The gene construct may contain a coding region which is identical or substantially similar to the nucleotide sequence as shown in SEQ-ID-NO-1 or SEQ-ID-NO2 or SEQ-ID-NO-3.

It is further preferred that the said sequence comprises a fragment being not less than 40 nucleotides capable of selective hybridisation to the endogenous plant cinnamoyl CoA reductase gene.

The invention further provides a plant transformed with a construct which is stably located within the genome of said plant.

In this specification "modulating the biomass" means increasing or decreasing the biomass relative to an untreated plant. It is preferred that any increase in biomass of plants be at least 5% of the plants total dry weight. The term "downregulation" means decreasing the level of expression of a gene already present in the plant.

In simple terms this invention requires the modulation of the expression of the endogenous plant cinnamoyl CoA reductase gene. Introduction of sense constructs or extra copies of CCR will result in either overexpression of the said gene providing plants having a reduced biomass, or a reduction in the activity providing an increase in biomass of the plant when compared with untreated or untransformed plants. In addition using the sequences in antisense orientation would provide plants having an increase in biomass when compared with untreated or untransformed plants.

An increase in plant biomass of crops is particularly advantageous when applied to plant species which are grown particularly for their biomass. Suitable examples include trees and principle fodder crops such as fescue, maize and fodder used for silage. It should also be noted that an increase in biomass would also be of benefit to the timber and paper industries as this would offer a greater quantity of renewable resources.

The invention also provides for other advantages associated with the suppression of the endogenous CCR enzyme by diverting substrate, normally used by CCR, to other biochemical pathways within the plant such as the production of plant pigments and defence related compounds such as phytoalexins.

Depending on the plant species to be transformed, a variety of different plant transformation vectors can be used. Preferred plasmids used in the construction of the plasmid used for transformations include pUC based vector systems available from Biolabs. The transformed cells may then in suitable cases be regenerated into whole plants in which the new genetic material is stably incorporated into the genome.
Examples of genetically modified plants which may be produced include but are not limited to field crops, fruit and vegetable such as: canola, sunflower, tobacco, sugarbeet, cotton, soya, maize, wheat, barley, rice, sorghum, tomatoes, mangoes, peaches, apples, pears, strawberries, bananas, melons, potatoes, carrot, lettuce, cabbage, asparagus, yams and onion.

The invention will now be described by way of examples wherein:
SEQ-ID-NO-1 is the tobacco cinnamoyl CoA reductase sequence.
SEQ-ID-NO-2 is the maize cinnamoyl CoA reductase sequence.
SEQ-ID-NO-3 is the eucalyptus cinnamoyl CoA reductase sequence.
SEQ-ID-NO-4 and SEQ-ID-NO-5 are PCR primer sequences.
FIGURE 1. Tobacco cinnamoyl CoA reductase construct.
FIGURE 2. Graphical representation of the tobacco stem average fresh weight in primary transformants exhibiting reduced cinnamoyl CoA reductase activity at 147 days post glasshouse acclimatisation.

FIGURE 3. Graphical representation of the tobacco stem average dry weight in primary transformants exhibiting reduced cinnamoyl CoA reductase activity at 147 days post transformation.

EXAMPLE 1.

Cloning and characterisation of cinnamoyl CoA reductase from tobacco.

A cDNA library from tobacco stem (Nicotiana tabacum cv Samsun), constructed in the Eco R1 site of the λZAPII vector (Stratagene, Cambridge, UK), was screened with a heterologous CCR probe from Eucalyptus. Approximately 400,000 plaques were screened and four positive clones were detected. They were excised into pBluescript (SK-) and further characterised by sequencing.

EXAMPLE 2.

Transformation and vector construction.

A 618bp CCR fragment encompassing a large portion of the coding sequence was amplified by PCR primers to the coding sequence with internal Xba 1 restriction sites (position 445bp to 1063bp). Forward primer (5'-tgtggtgtctagatcgtcaattgg-3') and reverse primer (5'-ttgagtaggatctagaaggtgac-3')(O' Connell et al., 1997 unpublished). The PCR product was restricted with Xba1 and cloned directly into Xba1restricted pJR1Ri, a Bin 19 derived vector containing the CaMV35S promoter and the 3' terminal end of the of the nopaline synthase gene (Smith et al, 1988).

EXAMPLE 3.

Plant transformation and regeneration.

The vector was transferred into Agrobacterium tumefaciens using a freeze thaw technique (An et al., 1988) and tobacco was transformed by a modification of the leaf disc method (Horsh et al., 1985). Kanamycin at 100mg/ml was used as selective agent and carbenicillen at 500mg/ml was used during the in-vitro regeneration procedure. The MS media was supplemented with 6-benzylaminopurine (6-BAP) (Sigma) 1mg/ml and naphthalene acetic acid (NAA) (Sigma) (0.1mg/ml). Kanamycin resistant shoots were selected after two round of screening on media containing kanamycin at 100mg/ml and carbenicillen at 200mg/ml minus 6-BAP and NAA. Duplicate plants were made from each shoot which were grown for 3-4 weeks.

EXAMPLE 4.

DNA analysis.

A 10cm disc of leaf tissue was taken from each reduced CCR line detected from the assay and used for the preparation of genomic DNA. The presence of the transgene was confirmed by the polymerase chain reaction (PCR) using primers to sequences as shown in example 2, the CaMV35S promoter and the 3' nopaline synthase terminator (Lassner et al., 1989) and transgene copy number was determined by southern blot analysis. Tobacco leaf genomic DNA was digested with Xba1 and separated on 0.8% agarose gels, using 20μg of DNA per lane. The DNA was transferred to Hybond-N membranes by alkaline transfer and fixed by baking for 2hrs at 80°C. The membranes were pre-washed at 65°C overnight in minimal hybridisation buffer (10% PEG, 7% SDS, 6XSSC, 10mM PO43- , 5mM EDTA, denatured sheared salmon sperm (100μg/ml)). The blot was hybridised overnight in the same fresh minimal hybridisation buffer with 32 p -labelled npt11 probe.

EXAMPLE 5.

The transformed primary transformant lines reduced in CCR activity were clonally propagated to provide several replicates of the same plant line. The experimental procedure was also repeated with the control plants. The calculation of biomass was performed by harvesting the stems of 3 replicates of each plant line at 147 days post acclimatisation in the glasshouse. The graphical representation, containing standard deviation error bars in Fig. 2., illustrates the average fresh weight of the transformed plants selected with a lower CCR activity as compared with an untransformed control. Plant line CCR86 shows a significant increase in stem fresh weight when compared with the control. The data is shown below in TABLE 1.

TABLE 1.

	Stem fresh weight (grams)		
plant line	average	sd (High)	sd (Low)
CCR47	119.93	131.4596	108.4004
CCR48	139.27	150.7328	127.8072
CCR83	130.5933	143.5035	117.6832
CCR88	142.9433	166.9883	118.8984
CCR49	133.5767	150.0133	117.14
CCR86	247.83	269.3226	226.3374
CCR57	119.38	126.9517	111.8083

CCR77	129.1467	148.7732	109.5201
CONTROL	112.62	132.1067	93.13327

"sd" means Standard deviation.

EXAMPLE 6.

The stem material from which the fresh weight was calculated and then lyophilised to enable the stem dry weight to be calculated. The graphical representation, containing standard deviation error bars in Fig. 3., illustrates the average dry weight of the transformed plants selected with a lower CCR activity as compared with an untransformed control. Plant lines CCR86 and CCR88 show significant increases in stem dry weight when compared with the control. The data is shown below in TABLE 2.

TABLE 2.

	Stem dry weight (grams)		
Plant line	average	sd(high)	sd(low)
CCR 47	23.07	25.49959	20.64041
CCR48	35.16333	38.89387	31.43279
CCR83	32.69667	37.12775	28.26558
CCR88	35.85333	41.9615	29.74516
CCR49	31.21	35.17228	27.24772
CCR86	44.23333	47.56221	40.90445
CCR57	28.11667	29.74965	26.48368
CCR77	28.65	33.56808	23.73192
CONTROL	22.92675	25.82365	20.02985

"sd" means Standard deviation.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: ZENECA LIMITED
(B) STREET: 15 STANHOPE GATE
(C) CITY: LONDON
(D) STATE: LONDON
(E) COUNTRY: UNITED KINGDOM
(F) POSTAL CODE (ZIP): W1Y 6LN
(G) TELEPHONE: 01344 414521
(H) TELEFAX: 01344 481112
(I) TELEX: 858270 ZENAGR G

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(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER:

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1293 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:

(B) CLONE: Tobacco Cinnamoyl CoA Reductase

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CCGAGCCTAT TTCTTCCCTA TATCCACTCA TCCTTGTCTT ATATCATCTT CATCATCATC 60

TACCTAAACC TGAGCTCAAC AGAAAAGTAA TACCATGCCG TCAGTTTCCG GCCAAATCGT 120

TTGTGTTACT GGCGCCGGAG GTTTCATCGC CTCTTGGCTC GTTAAAATTC TTCTGGAAAA 180

AGGCTACACT GTTAGAGGAA CAGTACGAAA TCCAGATGAT CGGAAAAATA GTCATTTGAG 240

GGAGCTTGAA GGAGCAAAAG AGAGATTGAC TCTGTGCAGA GCTGATCTTC TTGATTTTCA 300

GAGTTTGCGA GAAGCAATCA GCGGCTGTGA CGGAGTTTTC CACACAGCTT CGCCTGTCAC 360

TGATGATCCA GAACAAATGG TGGAGCCAGC AGTTATTGGT ACAAAGAATG TGATAACGGC 420

AGCAGCAGAG GCCAACGTGC GACGTGTGGT GTTCACTTCG TCAATTGGTG CTGTGTATAT 480

GGACCCAAAC AGGGACCCTG ATAAGGTTGT CGACGAGACT TGTTGGAGTG ATCCTGACTT 540

CTGCAAAAAC ACCAAGAATT GGTATTGCTA TGGAAAGATG GTGGCAGAAC AAGCAGCATG 600

GGACGAAGCA AGGGAGAAAG GAGTCGATTT GGTGGCAATC AACCCAGTGT TGGTGCTTGG 660

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CTCTGCTAAA ACATATGCCA ATTCAGTTCA GGCATATGTT CATGTTAGGG ATGTGGCTTT 780

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 713 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: Maize Cinnamoyl CoA Reductase

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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CGGGACAGAC AAAGGAGTAC GTCAACGAGT CGCACGCCTA CGTCGACGTC AGGGACGCCG 240

CCGAGGCGCA CGTCAGGGTG CTGGAGGCGC CCGGAGCCGG CGGCCGCCGG TACGTCTGCG 300

CCGAGCGCAC CCTGCACCGC GGCGAGCTCT GCCGCATCCT CGCCGGACTC TTCCCGGAGT 360

ACCCTATTCC GACAAGGTGC AAGGATCAGG TGAACCCACT GAAGAAGGGC TACAAGTTTA 420

CGAACCAACC TCTGAAGGAC CTTGGCGTCA AGTTCACGCC AGTTCATGGG TACCTGTACG 480

AAGCAGTGAA GTCCCTTCAA GACAAGGGGT TCCTCCCGAA GACATCTGGC GCCAAGGTGC 540

CTGAACGACG CAGCTGCCTG CCTCAAACGA CATCACAGCC ACCACCCGAA ATCGTTTCGA 600

AACTTTGAGG TGGATCTGCA CACGTGCTCA AACTGGCCAT GTGTTTTTTT GTCAGACAAG 660

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(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1297 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(vii) IMMEDIATE SOURCE:
(B) CLONE: Eucalyptus Cinnamoyl CoA Reductase

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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GCCGGCAATC CCACCATGCC CGTCGACGCC CTCCCCGGTT CCGGCCAGAC CGTCTGCGTC 180

ACCGGCGCCG GCGGGTTCAT CGCCTCCTGG ATTGTCAAGC TTCTCCTCGA GCGAGGCTAC 240

ACCGTGCGAG GAACCGTCAG GAACCCAGAC GACCCGAAGA ATGGTCATCT GAGAGATCTG 300

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TGATCTTAGA TCCATCACGG TGCGCATTTG TAATCCGGAG AAATGAGAGA AACATGTGGG 1200

AATTTGTTTG TACTTTTCTA AGTCAAACCT GGAGATACCA ACCCTGAGTT CTGCATTGGA 1260

ATGGAAGTTG TCAATTGTTC CAAAAAAAAA AAAAAAA 1297

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Polynucleotide Primer"

(vii) IMMEDIATE SOURCE:
(B) CLONE: Cinnamoyl CoA Reductase Forward Cloning Primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TGTGGTGTCT AGATCGTCAA TTGG 24

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Polynucletoide Primer"

(vii) IMMEDIATE SOURCE:
(B) CLONE: Cinnamoyl CoA Reductase Reverse Cloning Primer

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

TTGAGTAGGA TCTAGAAGGT GAC 23

CLAIMS

1. A method of modulating the biomass of a plant comprising altering the production or function of an endogenous plant cinnamoyl CoA reductase gene.

2. A method of modulating the biomass of a plant according to claim 1 comprising incorporating into the genome of a said plant a DNA which modulates the production or function of an endogenous plant cinnamoyl CoA reductase gene.

3. A method of increasing the biomass of plants according to claim 1, where said DNA has a substantially similar nucleotide sequence to an endogenous plant cinnamoyl CoA reductase gene.

4. A method according to claim 2 where said DNA is inserted into the plant in sense or antisense orientation.

5. A method according to any preceding claim where said DNA is a nucleotide sequence which is substantially similar to an endogenous plant cinnamoyl CoA reductase enzyme inhibitor.

6. A gene construct comprising in sequence a promoter which is operable in a target plant, a coding region which is substantially similar to an endogenous plant cinnamoyl CoA reductase gene and a termination sequence which is operable in a target plant.

7. A gene construct according to claim 5 where the promoter is switchable or inducible or tissue, organ or fruit specific.

8. A gene construct according to claim 6 where the promoter is cauliflower mosaic virus.

9. A gene construct further according to claim 5 where the coding region is identical or substantially similar to the nucleotide sequence as shown in SEQ-ID-NO-1.

10. A gene construct further according to claim 5 where the coding region is identical or substantially similar to the nucleotide sequence as shown in SEQ-ID-NO-2.

11. A gene construct further according to claim 5 where the coding region is identical or substantially similar to the nucleotide sequence as shown in SEQ-ID-NO-3.

12. A construct as claimed in any of the claims 8 to 10 where said sequence comprises a fragment being not less than 40 nucleotides capable of selective hybridisation to the endogenous plant cinnamoyl CoA reductase gene.

13. A plant transformed with a construct as claimed in any of claims 5 to 10 where said construct is stably located within the genome of said plant.

14. A plant according to claim 13 substantially as herein described.

15. A method according to any one of claims 1 to 5 substantially as herein described.

16. A gene construct according to any one of claims 6 to 12 substantially as herein described.

FIGURE 1

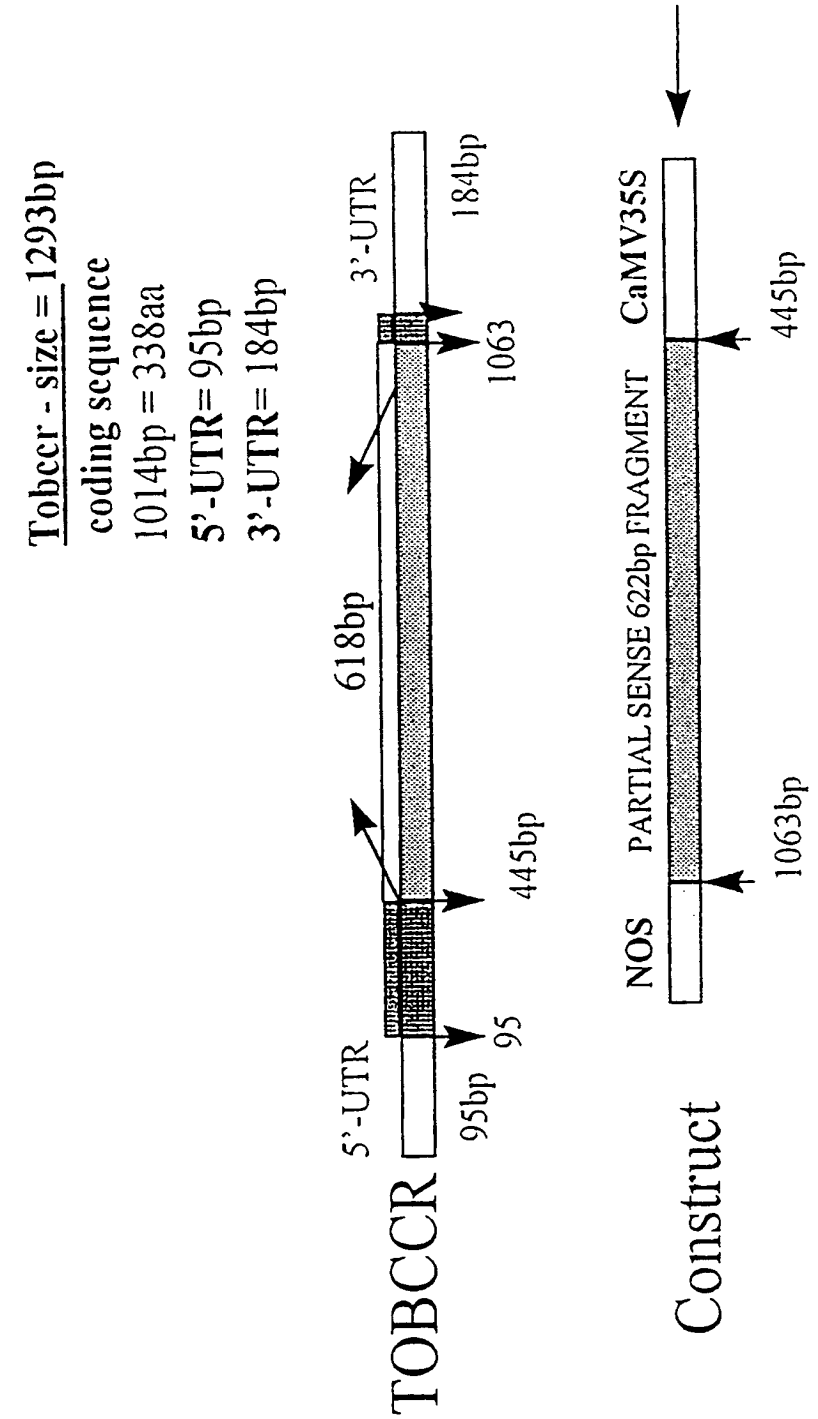


FIGURE 2

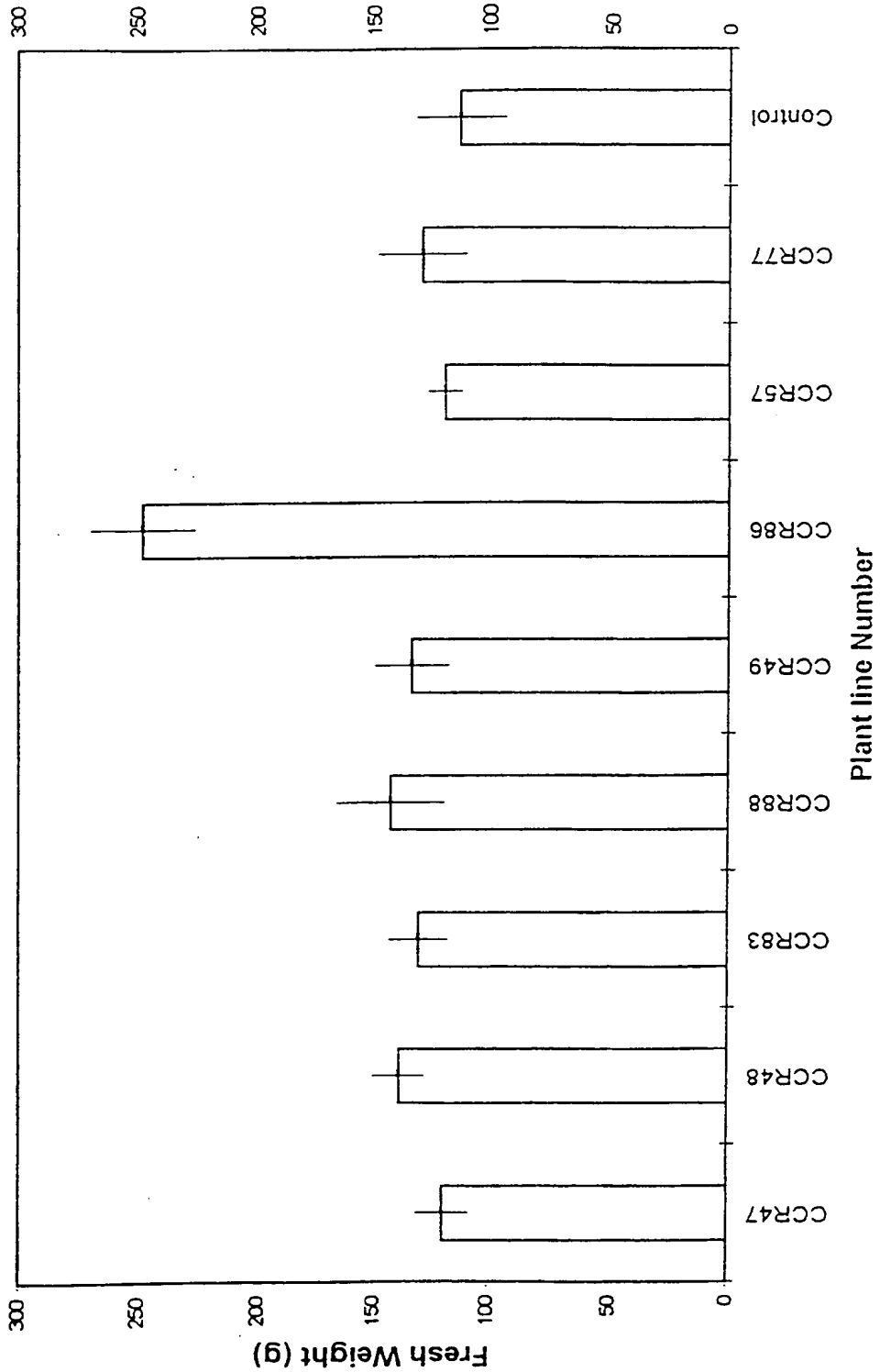
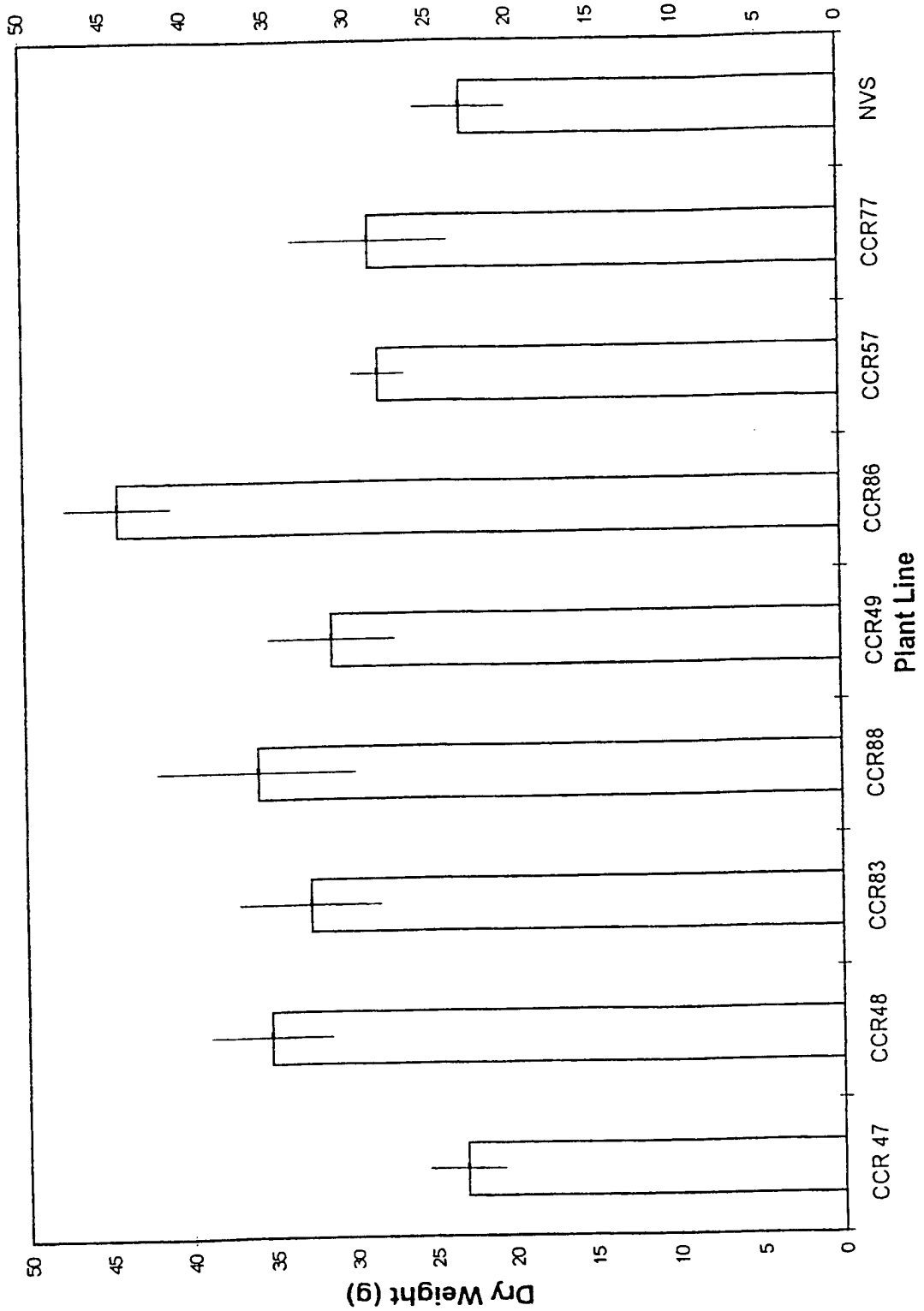


FIGURE 3



INTERNATIONAL SEARCH REPORT

Int. ...tion No
PCT/GB 98/00597

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 A01H1/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 05159 A (ICI PLC) 18 March 1993 see abstract see page 9, line 6 - page 11, line 8 see claims 1-10 ---	1,2,4, 6-8, 12-15
X	WO 95 27790 A (CENTRE NATIONATIONAL DE LA RECHERCHE SCIENTIFIQUE) 19 October 1995 see page 1, line 28 - line 35	1-9, 11-15
A	see page 4, line 9 - page 5, line 21 see claims ---	10
X,P	WO 97 12982 A (CENTRE NATIONA RECH SCIENT; INST NAT RECH AGRONOMIQUE) 10 April 1997 see the whole document ---	1-4,6-9, 11-15
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
17 June 1998	25/06/1998

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
E	WO 98 11205 A (FLETCHER CHALLENGE FORESTS LIM ;GENESIS RESEARCH & DEV CORP LI (NZ) 19 March 1998 see page 2, line 22 - page 4, line 23 see examples and claims	1-4,6-8, 12-16
X	BOUDET A. M. ET AL.: "LIGNIN GENETIC ENGINEERING" MOLECULAR BREEDING, vol. 2, 1996, pages 25-39, XP002025844 see abstract see page 32, column 1, line 3 - line 1	1,15
X	BOUDET A M ET AL: "TANSLEY REVIEW NO. 80 BIOCHEMISTRY AND MOLECULAR BIOLOGY OF LIGNIFICATION" NEW PHYTOLOGIST, vol. 129, no. 2, 1 January 1995, pages 203-236, XP002006037 see abstract see paragraph IV.2A see paragraph V	1-4,6, 13-16
A	GOFFNER D. ET AL.: "PURIFICATION AND CHARACTERIZATION OF CINNAMOYL-COENZYME A:NADP OXIDOREDUCTASE IN EUCALYPTUS GUNNII" PLANT PHYSIOLOGY, vol. 106, no. 2, 1 October 1994, pages 625-632, XP002006038	

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte[illegible]plication No
PCT/GB 98/00597

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9305159 A	18-03-1993	AU 669106 B	30-05-1996
		AU 1658192 A	05-04-1993
		BR 9205934 A	05-07-1994
		CA 2109222 A	27-10-1992
		EP 0584117 A	02-03-1994
		JP 6509465 T	27-10-1994
		US 5451514 A	19-09-1995
WO 9527790 A	19-10-1995	FR 2718460 A	13-10-1995
		AU 2347295 A	30-10-1995
		BR 9507291 A	23-09-1997
		CA 2185334 A	19-10-1995
		EP 0755449 A	29-01-1997
		JP 9511647 T	25-11-1997
		ZA 9502980 A	11-01-1996
WO 9712982 A	10-04-1997	FR 2739395 A	04-04-1997
WO 9811205 A	19-03-1998	AU 4403697 A	02-04-1998

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